Original articles

Epigenetic modification and uniparental inheritance of H19 in Beckwith-Wiedemann syndrome

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Abstract

Beckwith-Wiedemann syndrome (BWS) is a congenital overgrowth syndrome associated with a characteristic pattern of visceromegaly and predisposition to childhood tumours. BWS is a genetically heterogeneous disorder; most cases are sporadic but approximately 15% are familial and a small number of BWS patients have cytogenetic abnormalities involving chromosome 11p15. Genomic imprinting effects have been implicated in familial and non-familial BWS. We have investigated the molecular pathology of 106 sporadic BWS cases; 17% (14/83) of informative cases had uniparental disomy (UPD) for chromosome 11p15.5. In each case UPD appeared to result from a postzygotic event resulting in mosaicism for segmental paternal isodisomy. The critical region for isodisomy was refined to a 25 cM interval between D11S861 and D11S2071 which contained the IGF2, H19, and $p57^{KIP2}$ genes. In three cases isodisomy for 11q markers was detected but this did not extend further than 11q13-q21 suggesting that complete chromosome 11 disomy may not produce a BWS phenotype. The allele specific methylation status of the H19 gene was investigated in 80 sporadic BWS cases. All 13 cases with UPD tested displayed hypermethylation consistent with an excess of paternal H19 alleles. In addition, five of 63 (8%) cases with normal biparental inheritance had H19 hypermethylation consistent with an "imprinting centre" mutation (ICM) or "imprinting error" (IE) lesion. The phenotype of patients with putative ICM/IE mutations was variable and overlapped with that of non-UPD sporadic BWS cases with normal H19 methylation. However, exomphalos was significantly ($p<0.05$) more common in the latter group. These findings may indicate differential effects on the expression of imprinted genes in chromosome 11p15 according to the precise molecular pathology. Analysis of H19 methylation is useful for the diagnosis of both UPD or altered imprinting in BWS and shows that a variety of molecular mechanisms may cause relaxation of IGF2 imprinting in BWS.

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Genomic imprinting is a recently discovered mechanism by which the activity of genes may be altered epigenetically in a manner dependent on the parent of origin of each of the two alleles. Further work has implicated genes subject to imprinting in the control of embryonic growth and, of the genes now shown to be imprinted, eight have been shown to affect some aspect of cellular or organismal growth.[1] Imprinted genes tend to be clustered in specific chromosomal locations, and currently there is evidence that such imprinted loci may be controlled by *cis* acting "imprinting centres" which are involved either in the maintenance or initiation of the imprint.[2]

The observation that the penetrance of some human genetic diseases was subject to a non-X linked parent of origin effect led to the realisation that dysregulation of the normal pattern of imprinting in certain key genes may lead to hyperplastic/trophic or frank neoplastic disease.[3] Three human diseases, Angelman, Prader-Willi, and Beckwith-Wiedemann (BWS) syndromes have been extensively studied as paradigms of disordered genomic imprinting.[4]

BWS is a congenital overgrowth syndrome characterised by the association of gigantism, macroglossia, and visceromegaly with a variety of developmental anomalies such as exomphalos, umbilical hernias, hemihypertrophy, genitourinary abnormalities, and a predisposition to embryonal tumours.[5] The genetics of BWS are complex. Most cases are sporadic, but approximately 15% are familial and a small number have chromosomal aberrations of chromosome 11p. Clinical and molecular genetic studies suggest that the BWS gene, or

genes, are imprinted as, in affected families, penetrance is usually more complete with maternal inheritance.[6 7] Genomic imprinting effects have also been implicated in sporadic forms of the disease with a variety of mechanisms being implicated,[8-16] including (1) uniparental disomy for 11p, (2) paternally inherited duplications of 11p15, (3) maternally inherited balanced rearrangements of chromosome 11, and (4) putative "imprinting centre mutations" (ICM) or imprinting errors (IE), in which biallelic IGF2 expression is associated with hypermethylation of the IGF2 and H19 loci. IGF2 is paternally expressed in humans and mice, and while there is no evidence that circulating IGF-II is normally rate limiting on fetal growth,[17] situations in which it is deleted from the genome result in marked fetal growth reduction.[18] Analysis of tissue targeted expression of IGF-II suggests that local expression is a key factor in its biological effects.[19] A reciprocal phenotype is seen with the equivalent manipulations of the maternally expressed H19 gene which maps ~100 kb telomeric of IGF2. Regulation of IGF2 and H19 expression may be closely and reciprocally linked. Maternal inheritance of a deletion of the H19 gene and flanking sequences causes expression from the maternal IGF2 allele (which is normally silenced).[20]

In humans and mice, H19 and IGF2 show allele specific differences in DNA methylation,[11 21-25] and in previous studies we have shown an increase in DNA methylation at the IGF2/H19 loci in some sporadic BWS patients.[26] Initially hypermethylation was only detected in disomic cases, but recently a small subgroup of non-disomic BWS patients with IGF2/H19 methylation changes were identified.[16] These patients had the combination of a paternal methylation pattern on the maternal chromosome and biallelic IGF2 expression. It was proposed that these patients had mutations in a putative "imprinting centre" or errors in imprinting mechanisms. In addition, Morison *et al*[27] have reported three patients with somatic overgrowth and nephromegaly or Wilms' tumour who had biallelic IGF2 expression but did not satisfy the diagnostic criteria for BWS. All three cases had partial hypermethylation at H19.

In the light of increasing evidence for the heterogeneity of BWS, we have further investigated the origin, frequency, significance, and associated phenotype of H19 methylation abnormalities in 106 sporadic BWS patients, the largest sample reported to date. These studies confirmed the usefulness of H19 methylation analysis for the molecular diagnosis of BWS, identified a further two cases with an ICM/IE, and refined the critical region for disomy to a 25 cM interval between D11S861 and D11S2071.

Methods

PATIENTS

A total of 106 subjects (51 male, 55 female) with sporadic BWS were investigated. BWS was diagnosed according to previously defined criteria: (1) three major features (anterior abdominal wall defects, macroglossia and pre/postnatal growth >90th centile), or (2) two major features plus three or more of: characteristic ear signs (ear lobe creases or posterior helical ear pits), facial naevus flammeus, hypoglycaemia, nephromegaly, and hemihypertrophy.[4] All cases were sporadic with no family history of BWS, and only one patient had a cytogenetic abnormality, a male with a paternally derived duplication of 11p15.5. Peripheral blood for DNA analysis was obtained from each affected child and their parents. Of these patients, 49 were included in a previous study of uniparental disomy (UPD) in BWS[12] with 34 of these being part of an investigation into H19/IGF2 methylation.[26]

H19 METHYLATION ANALYSIS

High molecular weight DNA was isolated from peripheral blood by standard methods.[28] For analysis of H19 methylation, DNA samples (8-10 μg) were digested with a large excess of *Pst*I and *Sma*I as described previously.[26] After Southern analysis and hybridisation with a H19 cDNA probe, the H19 methylation status, as designated by methylation indices (MI), was assessed by densitometry of autoradiographs and comparing the ratio of the 1.8 kb (uncut methylated) and 1.0 kb (cut unmethylated) fragments for deviation from a 1:1 ratio. MI values of less than 0.6 had previously been considered to be the upper limit for a normal population.[26]

MOLECULAR GENETIC INVESTIGATION

All patients were screened for evidence of disomy at the tetranucleotide microsatellite polymorphism in tyrosine hydroxylase (TH) and *Apa*I RFLP within exon 9 of IGF2[28 29] in chromosome 11p15.5. TH was amplified in PCR reactions as reported previously.[11] To test for the *Apa*I RFLP site in the IGF2 gene DNA (100 ng) was amplified using 50 pmol primers (IGF2-F 5'-CTTGGACTTTGAGTCAAA-TTGG-3'; IGF2-R 5'-CCTCCTTTGGT-CTTACTGGG-3') in 25 μl mixtures containing 10 mmol/l Tris/HCl, pH 9.0, 50 mmol/l KCl, 1.5 mmol/l $MgCl_2$, 0.01 w/v% gelatin, 0.1 v/v% triton X-100, 250 μmol/l each dNTP, and 0.5 U *Taq* polymerase. Thermocycling conditions involved 30 cycles of 60 seconds denaturation at 92°C, 60 seconds annealing at 55°C, and two minutes for elongation at 72°C, concluding with a final extension period of five minutes.

To increase the informativeness for UPD detection, the extent of disomy was investigated using a series of microsatellite and RFLP markers from chromosome 11p and 11q. The *Rsa*I polymorphic fragment in exon 5 of H19 was amplified using 50 pmol of each primer (p3104F 5'-AGATTCAAAGCCTCCACGA-3'; p3461R 5'-AGTGTTTATTGATGAT-GAGTCCAG-3') in the same reaction mixture as detailed above with the addition of: 10% v/v DMSO, 10% v/v glycerol, and 0.05% w/v polyoxyethylene ether (W-1) (Sigma; Poole, Dorset). In this case, thermocycling involved an initial denaturation period of three minutes at 94°C followed by 30 cycles of 96°C for 30 sec-

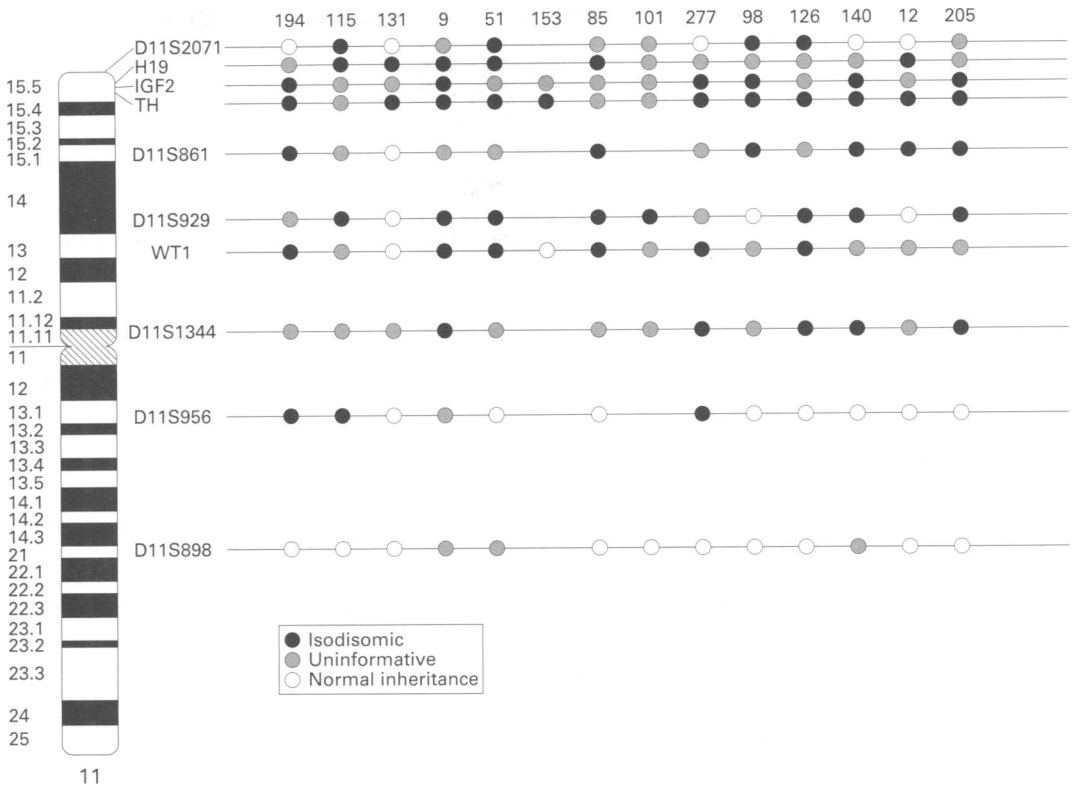

Figure 1 Map showing UPD in BWS patients as indicated by analysis of various chromosome 11 microsatellite (D11S-numbers) and RFLP polymorphic markers. Patient identification numbers are indicated at the top of each column. The H19, IGF2, and TH loci were within the disomic region in all cases and disomy extended onto 11q in three patients (194, 115, and 277).

onds, 52°C for 60 seconds, and 72°C for two mintes, concluding with a 10 minute extension at 72°C. Following the PCR of either IGF2 or H19 fragments, 10.5 ml product was digested with 30 U *Apa*I (10 U/μl) or *Rsa*I (10 U/μl) respectively in the appropriate reaction buffers according the supplier's conditions. Both digested and non-digested samples were electrophoresed through 2% agarose in TAE with DNA bands being visualised by ethidium bromide staining.

Amplification of the CA repeat within the WT1 gene (11p13) was performed essentially as previously outlined.[30] The dinucleotide repeat polymorphisms D11S861, D11S956, and D11S2017 were amplified under conditions described by Hudson *et al*,[31] Smith *et al*,[32] and Browne *et al*,[33] respectively. For analysis of the microsatellite polymorphisms at D11S929, D11S1344, and D11S898 the following primer sets were used: S929F 5'-AGG CCCTTCCAAGATCAG-3', S929R 5'-CCC AGTTGCCGAACTACC-3'; S1344F 5'-CCC TGAACTTCTGCATTCAC-3', S1344R 5'-GCGCCTGGCTTGTACATATA-3'; S898F 5'- AGCACCATTTGCTGAGACTG-3', S89 8R 5'-TGTATTTGTA- TCGATTAACCAA CTT-3'.[33] DNA (50 ng) was amplified in 13 μl reactions containing PCR buffer (10 mmol/l Tris/HCl, pH 9.0, 50 mmol/l KCl, 1.5 mmol/l $MgCl_2$, 0.01% w/v gelatin, 0.1% v/v triton X-100), 10 pmol of each primer, 125 μmol/l of each dNTP, and 0.25 U *Taq* polymerase. The samples were subjected to 30 PCR cycles of denaturation at 94°C for 45 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. Upon completion, a further five minutes 72°C extension was performed.

PCR products for each microsatellite marker were electrophoresed through 7.5 or 10% polyacrylamide (acrylamide:bis ratio 40:1, 1 × TBE) depending on the size of separation required. Gels were cast in a 20 cm × 20 cm × 1 mm vertical gel system and run in 1 × TBE. Samples (10 μl) were mixed with 3 μl (6×) gel loading dye (15% w/v ficoll, 5 mmol/l EDTA, 0.1% w/v SDS, 0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol) and electrophoresed at 100-120 volts for approximately 16 hours. Microsatellite bands were visualised by silver staining.

STATISTICAL ANALYSIS

Intergroup differences were compared using the chi-squared test with Yates' correction. Statistical significance was taken at the 5% level.

Results

UNIPARENTAL DISOMY IN BECKWITH-WIEDEMANN SYNDROME

A total of 69 of 83 (83%) cases informative at TH or IGF2 had normal biparental inheritance with no evidence of paternal UPD. No cases of heterodisomy were identified but 14 (17%) cases showed paternal isodisomy (figs 1 and 2). To investigate the origin and extent of paternal disomy a series of chromosome 11 polymorphic markers were analysed in these 14 cases. All showed mosaicism for partial paternal isodisomy suggesting that all UPD cases had arisen as a postzygotic event. The TH and

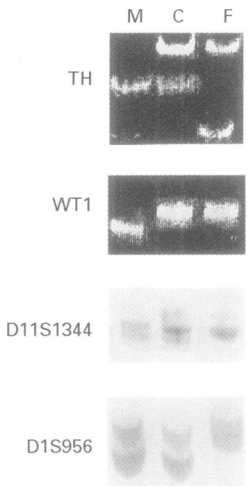

Figure 2 Molecular genetic analysis using chromosome 11 microsatellite markers and RFLPs. Inheritance of parental alleles in BWS patient 51, showing mosaicism. Tyrosine hydroxylase (TH) (11p15.5) and Wilms' tumour 1 (WT1) (11p13) are from ethidium bromide stained agarose gels, while D11S1344 (11p11) and D11S956 (11q13) are from PAGE gels stained with silver. M, mother; C, affected child; F, father.

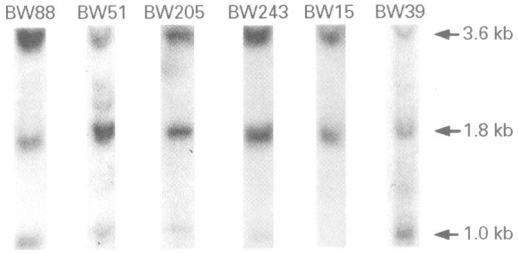

Figure 3 Increased H19 methylation in BWS patients. BW88 and BW39 are non-UPD patients with normal methylation patterns (MI=0.60 and 0.49 respectively). BW51 and BW205 were identified as UPD (fig 1) and show hypermethylation at the H19 locus. BW243 and BW15 represent BWS patients with normal inheritance of chromosome 11p15 alleles but have increased H19 methylation (MI=0.87 and 0.93 respectively) indicative of an ICM or IE lesion.

IGF2 loci were included in the minimally disomic region in all cases and the smallest region for disomy was a 25 cM region between D11S861 and D11S2071 (BW131) (fig 1). Disomy extended onto the long arm of chromosome 11 in three instances (BW194, BW115, BW277), but did not extend as far as 11q21 (D11S898) (fig 1).

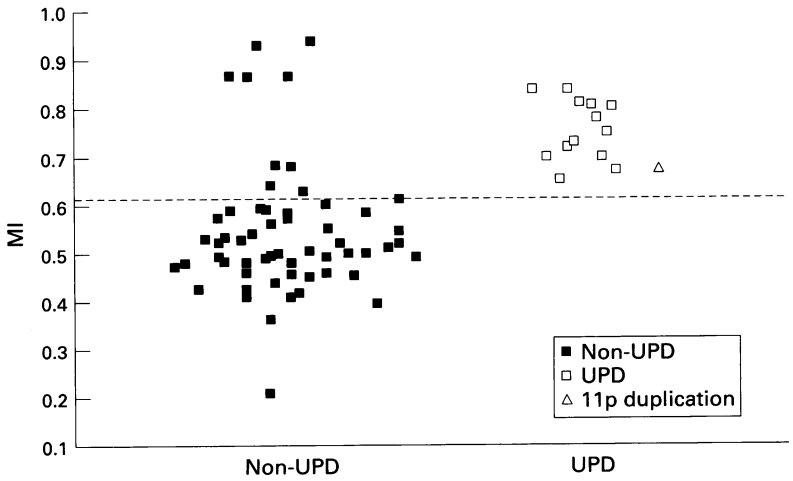

Figure 4 H19 methylation indices of BWS patients. MI were derived as described in Materials and methods. The patient represented by the triangle was shown to have a paternally derived duplication of 11p15.5 (MI=0.67).

H19 METHYLATION ANALYSIS

Allele specific methylation of the H19 promoter region was examined in the 80 BWS patient samples in which sufficient DNA was available for Southern analysis. Fig 3 illustrates typical examples; hypermethylation of the maternal allele of H19 is indicated by a reduction in intensity of the 1.0 kb digestion product. Methylation indices (MI) were derived from 63 patients informative for UPD analysis. In the 13 patients with paternal UPD analysed, the MI ranged between 0.65 and 0.84 and 40 patients, while most patients in whom paternal UPD had been excluded had a MI <0.64 (fig 4). A previously undescribed patient with paternally derived duplication of 11p15.5 had an MI of 0.67, consistent with a 2:1 ratio of paternal (methylated) to maternal H19 (unmethylated) alleles. Five patients with normal biparental inheritance, including three reported previously,[16] had a MI greater than those found in the UPD cases (>0.84).

CLINICAL PHENOTYPE OF BWS SUBGROUPS

In a previous study we had found that hemihypertrophy was significantly more common and exomphalos less frequent in UPD cases than in non-UPD patients.[11] The clinical phenotype of non-UPD patients with (MI>0.84, n=5) and without (MI<0.6, n=19) putative ICM/IE were compared. Postnatal growth >90th centile was similar in both groups (4/5 and 16/19 respectively); birth weight tended to be higher in cases with H19 hypermethylation (4/5 and 5/19 respectively had a birth weight >90th centile; $\chi^2 = 2.85$, $0.05<p<0.1$). In addition, exomphalos was less common in patients with ICM/IE (0/5 versus 13/19; $\chi^2 = 4.96$, $p<0.05$). Wilms' tumour occurred in 1/5 non-UPD patients with a MI>0.84, 0/19 non-UPD patients with MI<0.6, and 1/12 UPD patients.

Discussion

UNIPARENTAL DISOMY IN BECKWITH-WIEDEMANN SYNDROME

Uniparental disomy (UPD) in humans has been thought to be caused primarily by meiotic non-disjunction events followed by trisomy or monosomy "rescue". UPD has been reported for 16 human chromosomes and has indicated the likelihood of imprinting effects in certain genome regions.[34 35] The proportion of UPD identified (17%) in our informative BWS cases is comparable with that derived from smaller studies.[9 10 12] To date all BWS patients with UPD have had partial (segmental) and somatically mosaic paternal isodisomy. This is in contrast to paternally derived UPD in Angelman syndrome, which is usually complete and results from errors during meiosis. The absence of complete chromosome 11 isodisomy or heterodisomy could reflect the infrequency of trisomy 11 compared to trisomy 15, or more likely, that the presence of imprinted genes on chromosome 11q results in a non-BWS phenotype in patients with complete isodis-

omy. Hence, Webb *et al*[36] have reported a case of complete paternal chromosome 11 disomy as a result of trisomy 11 rescue which presented with prenatal growth retardation. Two loci for familial non-chromaffin paraganglioma (PGL1 and PGL2) have been mapped to chromosome 11q.[37 38] Familial paraganglioma only manifests when it is inherited from the father, suggesting that it is caused by mutations in an imprinted paternally expressed tumour suppressor gene. PGL2 maps to a 5 cM region of 11q13 between D11S956 and PYGM but three of our patients were disomic at D11S956 (fig 1). PGL1, however, maps to chromosome 11q21 which was beyond the limits of disomy in our BWS patients. No other imprinted genes have been mapped to 11q, but imprinted genes are frequently clustered and other genes may lie within this interval. The presence of imprinted genes distal to 11q13 may preclude the finding of complete paternal chromosome 11 disomy in people with a BWS phenotype.

Molecular genetic analysis of a BWS associated paternally derived duplication by Weksberg *et al*[14] showed that the duplicated region included genes distal to the calcitonin-A (CALCA) locus in 11p15.4. Analysis of the breakpoint regions of balanced translocations and pericentric inversions associated with BWS have indicated two breakpoint cluster regions, the most distal of which (BWSCR2) maps centromeric to a cluster of genes already known to be imprinted in the mouse, IGF2, H19, $p57^{KIP2}$, Ins, all of which are associated with embryonic growth. While IGF2 has been suggested as a candidate for the BWS locus, one familial study has reported exclusion of linkage to IGF2[39] and both biallelic and normal monoallelic IGF2 expression has been reported.[40] Bischoff *et al*[41] mapped the minimal disomic region in one BWS patient to an approximately 38 cM region between D11S922 (11p15.5) and D11S904 (11p14-p13). Our results refine the critical region for BWS still further to a 25 cM interval between D11S861 (11p15.1) and the 11p telomeric microsatellite marker D11S2071. This region contains both IGF2 and H19 and the BWSCR2 region defined by Redeker *et al*.[42]

H19 METHYLATION AND IGF2 EXPRESSION.

Biallelic IGF 2 expression has been found in tissues from BWS patients,[16 40] and is similarly dysregulated in Wilms' tumours. Inappropriate expression of IGF2 from the maternal allele has been shown to correlate with methylation of the promoter region of the neighbouring H19 gene in Wilms' tumour[43 44] and in BWS.[16] Hypermethylation of H19 is associated with transcriptional inactivity and hypermethylation of a region of exon 9 in the human IGF2 gene is associated with active transcription.[24] Removal of methylation by germline deletion of the DNA methylase enzyme results in IGF2 inactivity and transcription of the normally silent paternal H19 gene,[45] and deletion of the H19 gene results in IGF2 overexpression.[20] In addition, a small number of BWS patients (5-10%) have normal biallelic inheritance and H19 hypermethylation which exceeds that found in mosaic UPD patients. By analogy with similar findings in Angelman/Prader-Willi patients,[46] it has been proposed that these changes may result from alteration of an imprinting control region or imprinting errors.[16] In this context all five BWS patients identified so far have been sporadic cases, so there is, as yet, no definitive evidence as to whether possible ICM/IE lesions are genetic or epigenetic.

To date all three BWS patients with an ICM/IE who are informative for IGF2 polymorphic marker studies have shown biallelic IGF2 expression[16] (unpublished observations). However, the clinical phenotype of these patients is not uniform and although four had classical BWS features, one patient displayed an incomplete phenotype with mild macroglossia and an umbilical hernia but normal prenatal growth. Recently Morison *et al*[27] have described non-classical BWS overgrowth patients with biallelic IGF2 expression and H19 hypermethylation. However, the degree of hypermethylation was less than that seen in our ICM/IE patients. In addition to the five patients with markedly increased H19 hypermethylation (MI>0.84), several non-disomic patients had methylation indices slightly higher than the previously described upper limit of normal (0.6). Morison *et al*[27] suggested that patients with partial hypermethylation, such as these, may be mosaic for imprinting mutations. In addition to putative ICM or IEs causing aberrent H19/IGF2 methylation patterns and IGF2 overexpression, we have also detected biallelic IGF2 expression in some non-UPD BWS patients with normal H19 methylation (Joyce *et al*, submitted) and Brown *et al*[47] have described similar findings with normal H19 expression in a BWS family with a chromosome 11 inversion. These findings indicate that a variety of H19 dependent and independent mechanisms may lead to biallelic IGF2 expression in BWS.

Although biallelic IGF2 expression has been reported in complete and incomplete forms of sporadic BWS, normal imprinting of the IGF2 gene has also been reported,[40] perhaps indicating that loss of IGF2 imprinting is not the only mechanism from which a BWS phenotype can result. In addition to IGF2 and H19, other imprinted genes such as $p57^{KIP2}$ map to the minimally duplicated/disomic region.[48] The phenotype of BWS is variable and so the particular features may depend on the extent of disomy or the precise pathogenetic mechanism, if this resulted in differential changes in the expression of other imprinted genes.

PHENOTYPE/GENOTYPE CORRELATION

The identification of clear genotype/phenotype correlations would enhance clinical management and provide evidence to support the hypothesis that the precise BWS phenotype may be determined by the specific molecular pathology of BWS. Two studies have reported a high incidence of Wilms' tumour in BWS patients with UPD (40% and 50% respectively)[10 11] and our results continue to suggest that although Wilms' tumour may be

more common in children with BWS, the actual risk (~8%) is lower than previous estimates, which may have reflected an ascertainment bias. In addition, the occurrence of a Wilms' tumour in a non-UPD BWS patient shows that the tumour risk is not confined to BWS UPD patients. We have previously observed that hemihypertrophy was significantly more common and exomphalos was less common in the disomic group than the non-UPD group. These findings were attributed to the the mosaic nature of UPD, so it was surprising that there was evidence to suggest that exomphalos was less common in non-UPD cases with H19 hypermethylation than in those with normal methylation. This may indicate that exomphalos is not simply a consequence of increased intra-abdominal pressure owing to hypertrophy, but may involve the failure of specific processes such as cell adhesion or migration which could be affected by alterations in the activity of other imprinted genes at the locus. Further studies are needed to confirm these findings, to determine the risk of neoplasia in patients with ICM/IE, and to elucidate the mechanisms for these genotype-phenotype correlations. Knowledge of the imprinting status of genes such as H19 and $p57^{KIP2}$ in patients with UPD and ICM/IE compared to non-UPD without H19 methylation might provide insight into the cause of these associations. It is interesting to note that H19 expression is low or absent in the class of patients with a low incidence of exomphalos (that is, UPD and ICM/IE patients).

In summary, we have shown that H19 methylation analysis is a reliable method for detecting UPD in BWS patients and also detects small numbers of patients with "imprinting centre mutations" (5-10%). We studied methylation status in blood DNA and it is possible that H19 methylation may vary between tissues. However, to date, the results of H19 methylation analysis in cultured fibroblasts (n=3) from non-UPD patients is consistent with those obtained from blood DNA. The postzygotic origin of paternal UPD in BWS may indicate that complete isodisomy or heterodisomy of chromosome 11 has a low viability or non-BWS phenotype. Careful correlation of the clinical phenotype with the molecular pathology may provide an insight into the aetiology of the variable expression of BWS and determine what screening should be offered to BWS children. Definition of the critical region of chromosome 11p for UPD in BWS will provide a basis for understanding the aetiology of BWS, and the detailed comparison of the clinical features of BWS patients with variable lengths of segmental isodisomy may identify target regions for other imprinted genes on chromosome 11. Similarly, further studies of the phenotype and heterogeneity of molecular mechanisms in non-BWS UPD may provide critical insights into the role of IGF2, H19, $p57^{KIP2}$, and other imprinted genes in various facets of the BWS phenotype.

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